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Dual Immunofluorescence Study of Citrullinated Proteins in Alzheimer Diseased Frontal Cortex

Anthony P. Nicholas

Department of Neurology, University of Alabama at Birmingham, and The Birmingham Veterans Administration Medical Center, Birmingham, Alabama

Abstract

Deimination is a post-translational modification of proteins in which selected arginine amino acids are enzymatically converted to citrullines. Using dual-color immunofluorescence, the present study is the first to examine the frontal cortex of patients with Alzheimer's disease (AD) versus age-matched controls with an established monoclonal antibody (F95) against peptidyl-citrulline moieties. In AD specimens, a number of new findings were discovered, including evidence for deiminated proteins in extracellular plaques, the walls of large blood vessels, the nuclei of selective neurons immunoreactive for phosphorylated tau and numerous reactive astrocytes concentrated around extracellular plaques, ventricular surfaces and at the interface between the gray and white matter of the cortex. Although the identities of these citrullinated proteins remain largely unknown, the present study adds to the growing number of locations in which deiminated proteins may be found in the brains of patients with AD.

Keywords

amyloid beta; citrulline; deimination; glial fibrillary acidic protein; peptidyl arginine deiminase; tau

INTRODUCTION

Deimination is a calcium-mediated, post-translational modification in which arginine amino acids in selected proteins are converted to citrullines through the peptidyl arginine deiminase (PAD) family of enzymes. In the central nervous system, it was previously believed that the vast majority of protein deimination takes place in glial cells using the PAD-2 isozyme [2–4, 34], yet a number of studies now provide evidence that proteins in neuronal cells may also be deiminated [1, 3, 4, 18, 26, 27]. Our laboratory has shown that both myelin basic protein and glial fibrillary acidic protein (GFAP) are deiminated under non-pathologic conditions in rodent [27] and human brains [30]. However, there is mounting evidence to suggest that deimination is increased in various pathologic conditions affecting the nervous system, including glaucoma [7], prion disease [15, 16], hypoxia [3, 32], peripheral nerve injury [18], spinal cord neural regeneration [19], Parkinson's disease [26], Marburg disease [36], multiple sclerosis [23, 24, 29] and experimental autoimmune encephalomyelitis [12, 28, 31].

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*Corresponding author at: SC 360G2, 1720 2nd Avenue South, Department of Neurology, The University of Alabama at Birmingham, Birmingham, AL 35294, USA. Tel.: 205-934-8314; fax: 205-934-6678. nicholas@uab.edu (A.P. Nicholas).

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In Alzheimer's disease (AD), increased PAD-2 and deiminated protein immunoreactivities were shown in hippocampal astrocytes of human AD patients compared to non-demented elderly controls [14]. Using an antibody that recognizes peptidyl-citrulline moieties after they have been chemically modified, along with 2-dimensional gel electrophoresis and mass spectrometry, the first deiminated proteins identified as being increased in AD hippocampus were GFAP and vimentin [14]. In a more recent study using a different anti-citrulline antibody, penta-tricopeptide repeat domain-2 (PTCD2) was identified as another protein present in the citrullinated form in AD brains [1]. Additionally, both PAD-2 in astrocytes and PAD-4 in neurons were reportedly seen in AD hippocampus and entorhinal cortex [1]. In contrast, our laboratory used a well-established monoclonal antibody (referred to as F95) against peptidyl-citrulline [30] in the present study. The results of these experiments suggest that the number of deiminated proteins in AD brains should be expanded further.

MATERIALS AND METHODS

Formalin-fixed, paraffinized samples of frontal cortex were obtained post-mortem from the Brain Donation Program Bank associated with the Alzheimer's Disease Research Center at the University of Alabama at Birmingham (UAB). All patients that provided brain tissue did so with written, pre-mortem, informed consent in accordance with the Declaration of Helsinki. A total of 13 blocks were immunohistochemically examined, consisting of 5 age-matched controls without AD and 8 patients with a pre-mortem clinical diagnosis of AD that was verified post-mortem using established histopathological criteria [11].

These samples were cut (5 μ m) with an Microm HM 355S sliding microtome, adhered to plus-coated glass slides (Fisher) and deparaffinized in Citrisolv clearing agent (Fisher) and isopropanol. These sections were then prepared for antigen retrieval using citrate buffer (pH 6.0) steaming, followed by oxidation with 3% hydrogen peroxide. They were then incubated in auto-fluorescence eliminator solution (Sigma) containing Sudan black [6], followed by washes in 70% ethyl alcohol and phosphate buffered saline (PBS; pH 7.2). Any specimen still containing auto-fluorescence subsequently was not used.

All sections were then placed in a blocking solution containing 3% BSA and 5% non-fat powdered skim milk in PBS with 0.3% Triton X-100 and then incubated overnight at 4°C in 1:1000 dilutions of blocking solution containing the IgM F95 monoclonal antibody prepared from mouse ascites [18], or IgG mouse monoclonal antibodies against human amyloid beta peptide (A β) 1–42 (Signet), GFAP (Millipore), phosphorylated tau (P-tau; Millipore) or the neuronal marker NeuN (Pierce). The next day, sections processed only for F95 immunoperoxidase staining were washed with PBS, and then incubated in Immunopure Peroxidase Suppressor (Pierce) for 30 minutes followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Inc.) diluted 1:50 in PBS with 0.3% Triton X-100 for one hour at 37°C. The sections were then washed with PBS and placed in 0.1 M acetate buffer (pH 6.0). The slides were then incubated in a nickel-intensified diaminobenzadine (DAB) solution consisting of 0.035% DAB, 2.5% nickel ammonium sulfate and 0.01% hydrogen peroxide in acetate buffer for 5–15 minutes until immunoreactive structures stained black, with the optimum signal to noise ratio determined visually. Controls were performed in similar sections by eliminating the primary or secondary antibody.

For dual-color immunofluorescence, similar sections were instead incubated for one hour at 37°C in a 1:1000 dilution of blocking solution containing HRP-conjugated donkey secondary antiserum (Jackson ImmunoResearch). Tyramide signal amplification (TSA) was then employed (TSA Plus Fluorescence System, Perkin Elmer) utilizing a 30-minute incubation of TSA Plus Cy3 (1:500), resulting in F95 immunolabeled structures appearing

red. In contrast, A β , GFAP, NeuN and P-tau immunofluorescence utilized a 30-minute incubation of TSA Plus FITC (fluorescein isothiocyanate; 1:500), which appeared green.

All slides were then coverslipped with Vectashield[®] (Vector) and examined with a Nikon E-800 microscope. Digital images of this material were transferred to a Dell computer using a supercooled SPOT[™] color video camera (Diagnostic Instruments, Inc.). Adobe Photoshop CS software was used to save and merge the fluorescent images, as well as to optimize the contrast and brightness of the histological structures without altering the content.

RESULTS

Using nickel-intensified DAB as the chromogen and F95 as the primary antiserum, immunoreactive staining for citrullinated proteins in the frontal cortex of AD patients showed numerous patches of extracellular staining reminiscent of amyloid plaques (Fig. 1 A–B). Often surrounding these structures were cells with the morphology of reactive astrocytes (Fig. 1B). However, similar F95-stained cells were also found concentrated at the border between the grey and white matter (Fig. 1C) and no similar F95 staining was seen in age-matched controls (Fig. 1D).

Using dual-color immunofluorescence, F95 staining for deiminated proteins was often co-localized with immunoreactivity for human A β 1–42 in extracellular plaques (Fig. 2A) and within reactive astrocytes that were adjacent to small intraparenchymal blood vessels (Fig. 2B). Although F95 and A β immunoreactivities were also both observed in larger extraparenchymal blood vessels, little if any co-localization was seen in these structures (Fig. 2C).

When F95 immunofluorescence in AD frontal cortex was combined with that of GFAP (Fig. 3A–B), NeuN (Fig. 3C–D) or P-tau (Fig. 3E–F), additional observations were made. Evidence for deiminated GFAP in astrocytes was often seen (Fig. 3A–B), but the co-localization of F95 and GFAP was especially concentrated around extracellular plaques and at the ventricular surface (Fig. 3B). Reactive astrocytes containing deiminated protein were also concentrated in the AD frontal cortex just deep to the cortical ribbon (Fig. 3C–D). Although these cells and Neu-N positive neurons were closely adjacent, they primarily remained in separate cellular populations. Although in most instances deiminated proteins in AD frontal cortical cells were primarily seen in astrocytes, few exceptions were sometimes observed. For example, F95 immunoreactivity was occasionally seen in the cytoplasm (Fig. 3A) and nuclei (Fig. 3B, E–F) of cells that were not astrocytes. Using dual-color immunofluorescence of F95 and P-tau, no convincing co-localization was seen between these two immunoreactivities, although deiminated proteins appeared to be present in astrocytes surrounding neuritic plaques that also contained deiminated proteins (Fig. 3E). However, occasionally neurons that expressed immunofluorescence for P-tau in the cytoplasm, also expressed F95 immunoreactivity for deiminated proteins in their nuclei (Fig. 3F).

DISCUSSION

Including this report, there are now three studies examining deimination in AD brains and their collective findings are summarized in Table 1. The first experiments analyzed AD hippocampus versus age-matched controls [14] and utilized a rabbit IgG against peptidyl-citrulline after being chemically modified [33]. Often referred to as the "mod-cit" antibody, this immunoglobulin is commercially available (Upstate/Millipore). A second more recent study [1] examined both hippocampus and entorhinal cortex of AD patients and age-matched controls using another polyclonal IgG antibody against gluteraldehyde-conjugated

citrulline that is also commercially available (Abcam). However, using another, well-established IgM monoclonal antibody (referred to as F95) that reacts with multiple synthetic and naturally citrullinated proteins [30], the present study is the first to describe the anatomical locations of deiminated proteins in the frontal cortex of AD patients.

Although previous reports have shown that increased PAD-2 [1, 14] and protein deimination [14] are seen in large reactive astrocytes of human AD brains, the present study confirms these findings, but also provides further information regarding the location of these cells. For example, astrocytes containing deiminated proteins were observed around areas containing amyloid plaques [1, 14], but were also quite prominent at the junction between the gray and white matter of the frontal cortex, at the ventricular surface and engulfing small parenchymal blood vessels. Recently, immunoreactivities of GFAP, PAD-2 and citrullinated proteins were also observed in parenchymal blood vessels of both AD and age-matched control hippocampus and entorhinal cortex. In fact, co-localization of deiminated proteins with GFAP in astrocyte processes contacting parenchymal blood vessel walls has been seen previously in normal human brains [30], but the significance for these findings are still unknown.

Since it has been shown that deimination of GFAP and vimentin are increased in AD [14] and that this process results in dissociation and unfolding of these protein *in vitro* [13], perhaps deimination functions in some degree by changing astrocyte cell size or shape [38]. In this way, perhaps intermediate filament citrullination contributes in the ability of reactive astrocytes to wall off [38] and break up amyloid plaques in AD [35], thus contributing to the phagocytosis of extracellular proteins such as A β [25, 37] in an attempt to clear them from the brain by shuttling them to the bloodstream and cerebrospinal fluid [10, 37]. Certainly, this mechanism is supported by the present study, not only showing deiminated proteins and A β immunoreactivities in astrocytes that are in intimate contact with small parenchymal blood vessels, but also F95- and GFAP-positive fibers concentrated at the ventricular surface and surrounding amyloid plaques. In addition, the present study also documents the concentration of F95-positive astrocytes at the junction of the grey and white matter of AD frontal cortex, which was similar to that seen in the brains of patients with secondary progressive multiple sclerosis [29]. These findings, along with similar evidence for increased GFAP deimination in other neurodegenerative diseases [12, 15, 16, 26, 28, 29, 31], suggest that this reaction may be a non-specific response to some, but perhaps not all, types of brain damage. Obviously, further investigation on the true function of deiminated astrocyte filament proteins *in vivo* is still warranted.

The present study also demonstrates for the first time that larger extracerebral blood vessels in human AD brains contain deiminated proteins, although there did not appear to be any significant co-localization of F95 with A β , the latter of which being isolated to the muscular tunica media, as previously described [9, 17]. Certainly, future studies will focus on determining the identity of deiminated proteins in AD blood vessels and if citrullination contributes to the friability of vascular walls in AD angiopathy.

In addition, the present study shows evidence for deiminated proteins in extracellular plaques of AD frontal cortex. Although evidence for extracellular deiminated protein immunostaining in AD hippocampus was also shown in a previous report [14], the potential significance of this finding was not mentioned in that study. In any case, it should be stressed that although co-localization of F95 and A β immunoreactivities was seen in extracellular plaques and astrocytes in the present study, it cannot be guaranteed that this represents citrullinated A β , since some other protein also found in these locations may be deiminated instead.

It was once believed that all citrullinated proteins in the brain originated from the enzymatic activity of PAD-2 in glial cells [2–4, 34] and that deimination in neurons was nothing more than a post-mortem artifact [4]. However, there is growing evidence that this process may actually occur commonly in neurons [3, 4, 18, 26, 27], including recently, when evidence for citrullinated proteins and PAD-4 was demonstrated in human hippocampus and entorhinal cortical neurons of patients with AD, as well as age-matched controls [1].

In fact, a growing number of studies suggest that PAD-4 is the most likely enzyme to perform deimination in nuclei, since this process has been shown to citrullinate histone proteins of nuclear chromatin [8, 21], thus counteracting the post translational modification of arginine methylation [8]. The present study also provides evidence for nuclear deiminated proteins, especially in neurons expressing cytoplasmic immunoreactivity for P-tau, the latter of which is considered critically important in AD pathophysiology [5, 22]. In fact, the presence of neuronal nuclear deiminated proteins in AD may hold special significance, since post-translational modifications involved with epigenetic phenomenon are believed to play a critical role in memory formation in general [20]. In any case, collectively these studies suggest that, similar to glial cells, neuronal proteins may also be deiminated in AD, but the identity of the PAD isozymes that may perform this function, especially in different cellular compartments, is still in question.

CONCLUSIONS

The present study demonstrates a number of novel findings regarding the localization of deiminated proteins in AD frontal cortex, but also supports some past observations in other locations of AD brains using complimentary techniques (Table 1). As the list of potential deiminated proteins in AD grows, future studies will need to be employed, not only to identify all of these citrullinated proteins, but also to determine if any of them significantly contributes to AD pathophysiology and development of progressive dementia in this devastating disease.

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Abbreviations

AD	Alzheimer's disease
PAD	peptidyl arginine deiminase
GFAP	glial fibrillary acidic protein
PTCD2	penta-tricopeptide repeat domain-2
PBS	phosphate buffered saline
Aβ	amyloid beta peptide
P-tau	phosphorylated tau
HRP	horseradish peroxidase

DAB	diaminobenzadine
TSA	tyramide signal amplification
FITC	fluorescein isothiocyanate

REFERENCES

1. Acharya NK, Nagele EP, Han M, Coretti NJ, Demarshall C, Kosciuk MC, Boulos PA, Nagele RG. Neuronal PAD4 expression and protein citrullination: Possible role in production of autoantibodies associated with neurodegenerative disease. *J Autoimmun.* 2012; 38:369–380. [PubMed: 22560840]
2. Akiyama K, Inoue K, Senshu T. Immunocytochemical demonstration of skeletal muscle type peptidylarginine deiminase in various rat tissues. *Cell Biol Int Rep.* 1990; 14:267–273. [PubMed: 2188742]
3. Asaga H, Ishigami A. Protein deimination in the rat brain: Generation of citrulline-containing proteins in cerebrum perfused with oxygen-deprived media. *Biomedical Research.* 2000; 21:197–205.
4. Asaga H, Senshu T. Combined biochemical and immunocytochemical analyses of postmortem protein deimination in the rat spinal cord. *Cell Biol Int.* 1993; 17:525–532. [PubMed: 8339070]
5. Ballatore C, Lee VM, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci.* 2007; 8:663–672. [PubMed: 17684513]
6. Baschong W, Suetterlin R, Laeng RH. Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning microscopy (CLSM). *J Histochem Cytochem.* 2001; 49:1565–1571. [PubMed: 11724904]
7. Bhattacharya SK, Crabb JS, Bonilha VL, Gu X, Takahara H, Crabb JW. Proteomics implicates peptidyl arginine deiminase 2 and optic nerve citrullination in glaucoma pathogenesis. *Invest Ophthalmol Vis Sci.* 2006; 47:2508–2514. [PubMed: 16723463]
8. Cuthbert GL, Daujat S, Snowden AW, Erdjument-Bromage H, Hagiwara T, Yamada M, Schneider R, Gregory PD, Tempst P, Bannister AJ, Kouzarides T. Histone deimination antagonizes arginine methylation. *Cell.* 2004; 118:545–553. [PubMed: 15339660]
9. Frackowiak J, Zoltowska A, Wisniewski HM. Non-fibrillar β -amyloid protein is associated with smooth muscle cells of vessel walls in Alzheimer disease. *J Neuropathol Exp Neurol.* 1994; 53:637–645. [PubMed: 7964904]
10. Frautschy SA, Cole GM, Baird A. Phagocytosis and deposition of vascular beta-amyloid in rat brains injected with Alzheimer beta-amyloid. *Am J Pathol.* 1992; 140:1389–1399. [PubMed: 1376558]
11. Giannakopoulos P, Hof PR, Michel JP, Guimon J, Bouras C. Cerebral cortex pathology in aging and Alzheimer's disease: a quantitative survey of large hospital-based geriatric and psychiatric cohorts. *Brain Res Rev.* 1997; 25:217–245. [PubMed: 9403139]
12. Grant JE, Hu J, Liu T, Jain MR, Elkabes S, Li H. Post-translational modifications in the rat lumbar spinal cord in experimental autoimmune encephalomyelitis. *J Proteome Res.* 2007; 6:2786–2791. [PubMed: 17567059]
13. Inagaki M, Takahara H, Nishi Y, Sugawara K, Sato C. Ca^{2+} -dependent deimination-induced disassembly of intermediate filaments involves specific modification of the amino-terminal head domain. *J Biol Chem.* 1989; 264:18119–18127. [PubMed: 2808368]
14. Ishigami A, Ohsawa T, Hiratsuka M, Taguchi H, Kobayashi S, Saito Y, Murayama S, Asaga H, Toda T, Kimura N, Maruyama N. Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. *J Neurosci Res.* 2005; 80:120–128. [PubMed: 15704193]
15. Jang B, Jin JK, Jeon YC, Cho HJ, Ishigami A, Choi KC, Carp RI, Maruyama N, Kim YS, Choi EK. Involvement of peptidylarginine deiminase-mediated post-translational citrullination in pathogenesis of sporadic Creutzfeldt-Jakob disease. *Acta Neuropathol.* 2010; 119:199–210. [PubMed: 20013286]

16. Jang B, Kim E, Choi JK, Jin JK, Kim JI, Ishigami A, Maruyama N, Carp RI, Kim YS, Choi EK. Accumulation of citrullinated proteins by up-regulated peptidylarginine deiminase 2 in brains of scrapie-infected mice: a possible role in pathogenesis. *Am J Pathol.* 2008; 173:1129–1142. [PubMed: 18787103]
17. Kawai M, Kalaria RN, Cras P, Siedlak SL, Velasco ME, Shelton ER, Chan HW, Greenberg BD, Perry G. Degeneration of vascular muscle cells in cerebral amyloid angiopathy of Alzheimer disease. *Brain Res.* 1993; 623:142–146. [PubMed: 8221082]
18. Keilhoff G, Prell T, Langnaese K, Mawrin C, Simon M, Fansa H, Nicholas AP. Expression pattern of peptidylarginine deiminase in rat and human Schwann cells. *Dev Neurobiol.* 2008; 68:101–114. [PubMed: 17948239]
19. Lange S, Gögel S, Leung KY, Vernay B, Nicholas AP, Causey CP, Thompson PR, Greene ND, Ferretti P. Protein deiminases: new players in the developmentally regulated loss of neural regenerative ability. *Dev Biol.* 2011; 355:205–214. [PubMed: 21539830]
20. Levenson JM, Sweatt JD. Epigenetic mechanisms in memory formation. *Nat Rev Neurosci.* 2005; 6:108–118. [PubMed: 15654323]
21. Mastronardi FG, Wood DD, Mei J, Rajmakers R, Tseveleki V, Dosch H-M, Probert L, Casaccia-Bonnel P, Moscarello MA. Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: A role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. *J Neurosci.* 2006; 26:11387–11396. [PubMed: 17079667]
22. Mi K, Johnson GV. The role of tau phosphorylation in the pathogenesis of Alzheimer's disease. *Curr Alzheimer Res.* 2005; 3:449–463. [PubMed: 17168644]
23. Moscarello MA, Pritzker L, Mastronardi FG, Wood DD. Peptidylarginine deiminase: a candidate factor in demyelinating disease. *J Neurochem.* 2002; 81:335–343. [PubMed: 12064481]
24. Moscarello MA, Wood DD, Ackerly C, Boulias C. Myelin in multiple sclerosis is developmentally immature. *J Clin Invest.* 1994; 94:146–154. [PubMed: 7518827]
25. Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang H-Y. Astrocytes accumulate A β 42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. *Brain Res.* 2003; 971:197–209. [PubMed: 12706236]
26. Nicholas AP. Dual immunofluorescence study of citrullinated proteins in Parkinson diseased substantia nigra. *Neurosci Lett.* 2011; 495:26–29. [PubMed: 21414385]
27. Nicholas AP, King JL, Sambandam T, Echols JD, Gupta KB, McInnis C, Whitaker JN. Immunohistochemical localization of citrullinated proteins in adult rat brain. *J Comp Neurol.* 2003; 459:251–266. [PubMed: 12655508]
28. Nicholas AP, Sambandam T, Echols JD, Barnum SR. Expression of citrullinated proteins in murine experimental autoimmune encephalomyelitis. *J Comp Neurol.* 2005; 486:254–266. [PubMed: 15844173]
29. Nicholas AP, Sambandam T, Echols JD, Tourtellotte WW. Increased citrullinated glial fibrillary acidic protein in secondary progressive multiple sclerosis. *J Comp Neurol.* 2004; 473:128–136. [PubMed: 15067723]
30. Nicholas AP, Whitaker JN. Preparation of a monoclonal antibody to citrullinated epitopes: its characterization and some applications to immunohistochemistry in human brain. *Glia.* 2002; 37:328–336. [PubMed: 11870872]
31. Rajmakers R, Vogelzangs J, Croxford JL, Wesseling P, van Venrooij WJ, Pruijn GJ. Citrullination of central nervous system proteins during the development of experimental autoimmune encephalomyelitis. *J Comp Neurol.* 2005; 486:243–253. [PubMed: 15844172]
32. Sambandam T, Belousova M, Accavitti-Loper MA, Blanquicett C, Guercello V, Rajmakers R, Nicholas AP. Increased peptidylarginine deiminase type II in hypoxic astrocytes. *Biochem Biophys Res Comm.* 2004; 325:1324–1329. [PubMed: 15555572]
33. Senshu T, Sato T, Inoue T, Akiyama K, Asaga H. Detection of citrulline residues in deiminated proteins on polyvinylidene difluoride membrane. *Anal Biochem.* 1992; 203:94–100. [PubMed: 1524220]
34. Vincent SR, Leung E, Watanabe K. Immunohistochemical localization of peptidylarginine deiminase in rat brain. *J Chem Neuroanatomy.* 1992; 5:159–168.

35. Wegiel J, Wang KC, Tarnawski M, Lach B. Microglia cells are the driving force in fibrillar plaque formation, whereas astrocytes are a leading factor in plaque degradation. *Acta Neuropathol.* 2000; 100:356–364. [PubMed: 10985692]
36. Wood DD, Bilbao JM, O'Connors P, Moscarello MA. Acute multiple sclerosis (Marburg type) is associated with developmentally immature myelin basic protein. *Ann Neurol.* 1996; 40:18–24. [PubMed: 8687186]
37. Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, Husemann J. Adult mouse astrocytes degrade amyloid- β in vitro and in situ. *Nature Med.* 2003; 9:453–457. [PubMed: 12612547]
38. Xu K, Malouf AT, Messing A, Silver J. Glial fibrillary acidic protein is necessary for mature astrocytes to react to beta-amyloid. *Glia.* 1999; 25:390–403. [PubMed: 10028921]

Highlights

Increased deiminated proteins were seen in astrocytes of Alzheimer frontal cortex.

Increased deiminated proteins were seen in Alzheimer brain blood vessels.

Increased deiminated proteins were seen in Alzheimer amyloid and neuritic plaques.

Deiminated proteins were seen in nuclei of phosphorylated tau-labeled neurons.

Fig. 1.

Deiminated protein immunoperoxidase staining using the F95 antibody alone demonstrated numerous extracellular plaques in AD frontal cortex (A). In higher magnification (B), many of these were surrounded by F95-positive cells with the morphology of reactive astrocytes (arrows). Similar cells were also seen at the interface between the grey and white matter in AD patients (C, example magnified in insert), but not in age-matched controls (D). Scale bars = 50 μ m.

Fig. 2. Co-localization (yellow) of F95 (red) and A β (green) immunoreactivities in AD frontal cortex was seen in extracellular plaques (A) adjacent to astrocytes containing deiminated protein (asterisk) and within astrocytes (B) that were in intimate contact with small intraparenchymal blood vessels (arrow). In larger extraparenchymal blood vessels (C), no co-localization was seen between immunoreactivities for F95 versus A β , with the latter primary located in tunica media (insert). Scale bars = 50 μ m.

Fig. 3.

In AD frontal cortex, co-localization (yellow) of immunofluorescence for F95 (red) with other antigens (green) such as GFAP (A–B), NeuN (C–D) or P-tau (E–F), demonstrated numerous novel findings. For example, many astrocytes were shown containing deiminated proteins (yellow cells in A–B, red cells in C–E), especially at the ventricular surface (top in B), surrounding circumscribed extracellular plaques (arrows in B, E) and concentrated at the interface (white diagonal line in C) between the gray (top left) and white matter (bottom right) adjacent to NeuN-positive neurons (green cells in C–D). However, not all astrocytes contained deiminated proteins (white arrows in A) and deiminated proteins were also found in the cytoplasm (red arrow in A) and nuclei (red arrows in B) of cells that were not astrocytes. Combined with immunofluorescence for P-tau (green, E–F), deiminated proteins (red) were also found in reactive astrocytes (asterisks in E) surrounding the cores of neuritic plaques (arrow in E) and within nuclei (arrows in F) of neurons immunoreactive for P-tau. Scale bars = 50 μm.

Table 1

Evidence for deiminated proteins in Alzheimer brain astrocytes (A), blood vessels (B), neurons (N) and extracellular (E) locations

Ishigami et al, 2005: (hippocampus compared to age-matched controls)				
1) Increased PAD-2 in AD astrocytes	A			
2) Increased deiminated proteins in AD astrocytes	A			
3) Increased deiminated GFAP and vimentin in AD protein extracts	A			
4) Increased extracellular deiminated proteins in AD (present in figures, but not mentioned in text)				E
Acharya et al, 2012: (hippocampus and entorhinal cortex compared to age-matched controls)				
1) Increased PAD-2 in AD astrocytes	A			
2) Deiminated proteins in most AD and age-matched control astrocytes	A			
3) GFAP and PAD-2 in AD and age-matched control blood vessels	A	B		
4) Deiminated proteins in most AD and age-matched control neurons			N	
5) PAD-4 in most AD and age-matched control neurons			N	
6) PAD-4 and beta amyloid in many AD neurons			N	
7) Increased deiminated PTCD-2 in human AD protein extracts	Exact localization not established			
The present study (frontal cortex compared to age-matched controls):				
1) Increased deiminated proteins in AD astrocytes (especially around plaques, the ventricular surface and the junction between the cortical gray and white matter)	A			
2) Increased deiminated proteins in small parenchymal AD blood vessels		B		
3) Increased deiminated proteins in large periventricular AD blood vessels (but not co-localized with Aβ in the tunica media)		B		
4) Deiminated proteins in the nuclei of some AD neurons that expressed phosphorylated tau in the cytoplasm			N	
5) Co-localization of deiminated proteins and Aβ in AD astrocytes engulfing small parenchymal blood vessels	A	B		
6) Co-localization of deiminated proteins and Aβ in extracellular AD plaques				E
7) Deiminated proteins in extracellular AD neuritic plaques				E